





Article

# Combined Effects of Compost and *Medicago Sativa* in Recovery a PCB Contaminated Soil

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**Abstract:** The effectiveness of adding compost and the plant *Medicago sativa* in improving the quality of a soil historically contaminated by polychlorinated biphenyls (PCBs) was tested in greenhouse microcosms. Plant pots, containing soil samples from an area contaminated by PCBs, were treated with the compost and the plant, separately or together. Moreover, un-treated and un-planted microcosms were used as controls. At fixed times (1, 133 and 224 days), PCBs were analysed and the structure (cell abundance, phylogenetic characterization) and functioning (cell viability, dehydrogenase activity) of the natural microbial community were also measured. The results showed the effectiveness of the compost and plant in increasing the microbial activity, cell viability, and bacteria/fungi ratio, and in decreasing the amount of higher-chlorinated PCBs. Moreover, a higher number of  $\alpha$ -*Proteobacteria*, one of the main bacterial groups involved in the degradation of PCBs, was found in the compost and plant co-presence.

**Keywords:** plant-assisted bioremediation; microbial structure; microbial functioning; persistent organic pollutants; ELFA; FISH methods

## 1. Introduction

Soil microorganisms provide regulating ecosystem services, playing a central role in ensuring the quality of ecosystems. The presence of a rich and diverse microbial community makes it possible to recover soil from contamination [1,2]. However, soil remediation is possible if the detrimental effects of chemicals do not kill or inhibit microbial populations [3]. Particular concern arises from polychlorinated biphenyls (PCBs) which are persistent organic pollutants (POPs), as classified in the Stockholm Convention in 2001, and exclusively generated from anthropogenic sources. Although they have been prohibited since the 1970s, these POPs have been causing continuous exposition of water, air, sediments, soil and living organisms [4].

The most common PCB remediation strategies implemented today are “dig and dump” and “dig and incinerate” [5], but there is a need to apply effective green remediation strategies in line with environmental sustainability guidelines [6].

Laboratory experiments have recognized several bacterial pathways of PCB transformation. Lower halogenated PCBs (Cl < 4) can be transformed by aerobic degradation. Higher halogenated PCBs (Cl > 4) can be reductively de-chlorinated in anaerobic conditions when organic compounds are present [7]. However, the occurrence of these bio-transformations is difficult to achieve in one environment.

The biostimulation of PCB degradation can occur by adding compost to contaminated soil, because organic carbon sources can substantially increase their dechlorination [8]. Moreover, plants can help natural microorganisms in transforming, removing, and containing contaminants, including PCBs, in the plant-assisted bioremediation [9–13]. Inside the rhizosphere, microbial activity is promoted by root exudates, which comprise plant secondary metabolites that act as chemical signals and can stimulate the bacterial enzymes involved in PCB transformation [14–16]. In exchange, microorganisms produce molecules which are able to suppress plant pathogens or act as antibiotics [17,18]. Moreover, by exploring soil with their oxygen-transporting roots, plants offer the chance of exposing anoxic portion of soil to aerobic conditions, favouring the PCB oxidation with less than five chlorine atoms. Among plants identified for promoting PCB dissipation there are the *Populus* and *Salix* trees and the forage *Medicago sativa* [19–23]. *M. sativa* (alfalfa) is the primary, widely grown perennial legume in the world, a nitrogen fixing plant that has been found to help bacterial communities involved in PCB degradation [24–27]. Although adding compost and plants can improve in different ways the soil quality status, the pathways of PCB degradation, the environmental factors involved in their transformations, and the rhizosphere microbial community remain to be explored [28].

The objective of this research was to evaluate the effects of two treatments (i.e. biostimulation and plant-assisted bioremediation) in improving the soil quality and biodegradation of PCBs in microcosm experiments. For this purpose, a municipal solid waste compost and *Medicago sativa* seeds were added to soil samples collected from a PCB historically contaminated area. Soil microcosms with compost and/or plants were compared with respective untreated soil controls. The experimental set was maintained in a greenhouse for more than seven months. At fixed times, eighteen PCB congeners (six markers and twelve dioxin-like) were searched for. Moreover, the changes in the microbial community in terms of abundance, activity and composition were assessed.

## 2. Materials and Methods

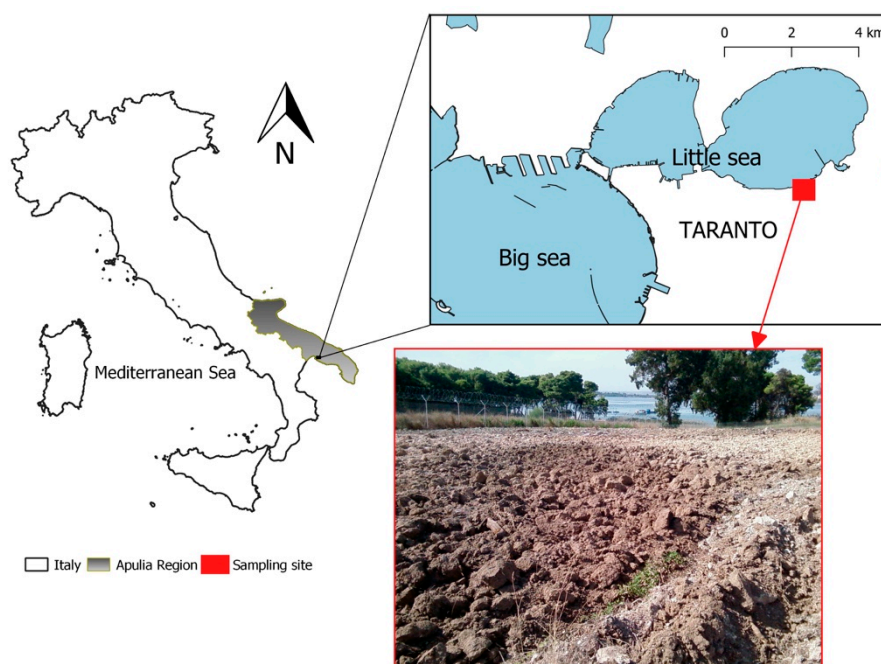
### 2.1. Soil Collection and Compost Description

Soil was gathered from a PCB contaminated site, located in Apulia Region (Southern Italy), overlooking the Little Sea of Taranto, where a phytoremediation strategy, applying the Monviso poplar clone (*Populus generosa* × *Populus nigra*) as the main detoxifying plant, has been in progress for more than 4 years (Figure 1). The soil was previously analysed by the local Region Environmental Agency and a PCB concentration exceeding the National legal limit (60 ng g<sup>-1</sup> for gardens, parks and residential areas, Italian D.Lgs. 152/06) was found in several points, with the highest values of 220 ng g<sup>-1</sup> [10].

It was decided to sow *Medicago sativa* between poplar tree rows to improve soil recovery in terms of both fertility and PCB decontamination. An experiment with laboratory microcosms was set up to verify this strategy under controlled conditions.

The samples consisted of equal proportions of surface soil (0–20 cm) collected in three points in which PCB contamination was above the legislative limit of 60 ng g<sup>-1</sup>. Soil samples were prepared by eliminating stones and residues of plant roots dried at ambient temperature and sieved (2-mm mesh).

The main characteristics of the soil mix were soil pH (in water): 8; organic carbon content: ca. 1.5%; total nitrogen: 0.02%. The soil was a sandy loam in accordance with the USDA (United States Department of Agriculture) classification of particle-size fractions ([https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/survey/?cid=nrcs142p2\\_054167](https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/survey/?cid=nrcs142p2_054167)). The municipal solid waste compost applied to the microcosm experiment was ISO/IEC 17025 certified. It was provided by the Italian Composters Consortium [29]. The microbiological characterization of the compost is reported in Table 1.



**Figure 1.** Location of the sampling site overlooking the Little Sea of Taranto.

**Table 1.** Municipal solid waste compost: microbiological parameters analysed in accordance with the legislation for fertilizers (Italian Decree 75/2010) for pathogens (*E. coli* and *Salmonella*) and additional ones (Microbial abundance, Cell viability and Dehydrogenase activity) assessed for its initial characterization before the addition to soil microcosms.

		Italian Legal Limits
<i>E. coli</i> (CFU g <sup>-1</sup> )	<25	≤1000
<i>Salmonella</i> (CFU g <sup>-1</sup> )	Absent	Absent
Microbial abundance (No. cells g <sup>-1</sup> )	1.0 × 10 <sup>10</sup>	-
Cell viability (% live cells/live + dead)	3%	-
Dehydrogenase activity (μg TPF g <sup>-1</sup> )	550	-

## 2.2. Microcosm Set up

Each plant pot microcosm (1 L) was filled with about 800 g of the composite soil. In twelve microcosms, only the historically contaminated soil was added. In another twelve pots, the same soil was previously mixed with compost (with a ratio 0.1 compost to 1 soil), which represented an agronomic compost dose of 26 t ha<sup>-1</sup>.

Lastly, Alfalfa seeds were sowed in half of the microcosms. Alfalfa was chosen for both its potential to enhance PCB dissipation and its ability to improve soil quality, being a nitrogen-fixing plant. For each different condition (i.e. HCS +/- plant and Compost +/- plant) six replicates (twenty-four microcosms in total) were performed. The experimental set was maintained for 224 days in a greenhouse (18–22 °C).

The four experimental conditions are summarized as follows:

- Historically contaminated soil = HCS;
- Historically contaminated soil + Alfalfa = Plant;
- Historically contaminated soil + Compost = Compost;
- Historically contaminated soil + Compost + Alfalfa = Compost + Plant

The soil humidity was monitored in order to maintain it to ca. 60% of its field capacity. At each sampling, the microcosms (two replicates for each condition) were destructively collected. Each microcosm was divided in two parts: one was immediately processed for analysing the microbial cell

abundance, viability and dehydrogenase activity at 1, 133 and 224 days. The microbial structure was also evaluated by the fluorescence in situ hybridization (FISH) technique at the start and at the end of the experiment. The other part of soil was frozen ( $-20\text{ }^{\circ}\text{C}$ ) for the subsequent extraction of PCBs and ester-linked fatty acid (ELFA) analyses. Similar to FISH, the total ELFAs of the overall microbial community (including Fungi) was performed at 1 and 224 days.

At 133 and 224 days, when the seeds had grown and the roots completely filled the pots, soil and plants were sampled. In particular, roots and leaves were washed as described by Di Lenola et al. [29]. This washing step was performed to ensure that only the PCBs absorbed by the plant were measured. The water content of the plant tissue was measured by weighing fresh leaves (10 g) and roots (1 g), which were then oven-dried at  $60\text{ }^{\circ}\text{C}$  for 3 days.

### 2.3. Chemical Analysis of Specific PCB Congeners

The PCB congeners analysed were the six markers (28, 52, 101, 138, 153, and 180) and the twelve dioxin-like (77, 81, 105, 118, 114, 123, 126, 156, 157, 167, 169, and 189), as recommended by EU Regulation No. 1259/2011. The first six PCBs are the most commonly found in the environment and represent those generally accumulated after a first biodegradation step. The second group of twelve PCBs, displaying toxicological properties similar to dioxins, can be harmful to health if found in feed and food. The congener numbers used are those defined by Ballschmiter and Zell [30].

The quantitative analyses of PCB congeners in soil and plant tissues were performed in accordance with the EPA method 1668. PCB extractions were performed by ASE (Accelerated Solvent Extraction) adopting the procedure reported in Thermo Scientific Technical note (210). Soil and plant tissue extracts were subsequently analysed using a Gas Chromatography coupled with a mass spectrometry (Thermo Scientific Finnigan TRACE GC ultra), as described in detail by Ancona et al. [10].

The PCB degradation (%) in soil was calculated for each PCB isomeric class/group (PCBs with the same chloride numbers) in accordance with how reported in Di Lenola et al. [29].

The bioaccumulation of PCBs in the plant roots and shoot tissues was calculated using the bioaccumulation factor ( $\text{BAF}_{\text{plant part}} = [\text{PCB}]_{\text{plant part}}/[\text{PCB}]_{\text{soil}}$ ) for each PCB congener found [31].

### 2.4. Microbiological Analyses

Microbial abundance and cell viability were analysed using direct counting methods with an epifluorescence microscope (Leica DM4000B). The microbial abundance (No. cells  $\text{g}^{-1}$  dry soil) was assessed with DAPI stain as the binder of the double-stranded DNA, in fixed soil aliquots. Soil subsamples (1 g) from each microcosm were processed as reported in detail in Barra Caracciolo et al. [32].

The cell viability (% live cells/live + dead) was evaluated in fresh soil subsamples (1 g). The two dyes used to measure the ratio of live to dead cells were propidium iodide and SYBR Green II (Sigma-Aldrich, Germany) respectively, as described in Grenni et al. [33,34].

Finally, the microbial activity ( $\mu\text{g TPFg}^{-1}$  dry soil) in soil samples was measured with the dehydrogenase assay. This method measures the red-coloured formazan (TPF) produced from the microbial reduction of 2,3,5- triphenyltetrazolium chloride (TTC). Soil sub-samples (5 g) were incubated at  $37\text{ }^{\circ}\text{C}$  in the dark for 24 h, and the TPF was colorimetrically determined at 485 nm with a spectrophotometer (Perkin-Elmer Lambda 40 UV/VIS spectrophotometer). Details of the method are reported in Grenni et al. [33,34].

### 2.5. Structure of Microbial Community Assessed by ELFAs

The ester-linked fatty acid (ELFA) analysis, one of the most successful biochemical methods for assessing microbial profiling and obtaining a taxonomic fingerprint of the main microbial groups (Gram-positive, Gram-negative bacteria and Fungi) occurring in a natural microbial community [1,35], was applied to soil sub-samples (3 g).

The relative abundance of fatty acids found in the different microcosm conditions was expressed as the mole percent (Mol %) of total fatty acids. The ELFA 18:2 $\omega$ 6,9c was used as the biomarker for

Fungi, the 16:1 $\omega$ 7; 18:1 $\omega$ 7c and 19:0cy for Gram-negative Bacteria and, finally, the i15:0, a15:0, i16:0 and i17:0 for Gram-positive Bacteria [36,37]. This method was applied following the procedure described by Schutter and Dick [38] and Di Lenola et al. [29].

### 2.6. Microbial Community Structure Assessed by FISH

The fluorescence in situ hybridization (FISH) method was used for identifying under the epifluorescence microscope the structure of the active microbial community [32,39,40]. FISH was applied on fixed soil sub-samples (1 g), from each replicate microcosm, following the procedure described in detail in Barra Caracciolo et al. [41]. The fluorescent oligonucleotide probes applied were those for *Archaea* and *Bacteria*, and within the latter, those of the most frequently groups found in soil [39]. The list of the probes applied is shown in Table 2; they were labelled with a Cy3 or FAM fluorochrome at the 5' end. The probe details are reported in Greuter et al. [42]. The evaluation of cells bound to the fluorescent probes was calculated as a percentage of the total DAPI positive cells (% positive cells vs DAPI) and as the number of positive cells (No. positive cells g<sup>-1</sup> soil).

### 2.7. Statistical Analysis

Each chemical and microbiological datum is the average value of the analysis performed in the two replicate microcosms per each sampling. The statistical analysis for assessing differences in PCB concentration (of total PCBs and of specific congeners), dehydrogenase activity and microbial abundance during the experimental period was performed using the two-way ANOVA analysis, with significant differences at the  $p < 0.05$  level. The time was always considered as variable 1 and the plant or compost occurrence as variable 2. The statistical analysis of ELFA and FISH data was achieved using the one-way ANOVA with treatment as the experimental factor. The statistical analysis Software used was SigmaStat.

## 3. Results

### 3.1. Chemical Analysis

The results of the chemical determinations of PCBs (ng g<sup>-1</sup> soil) in the different soil microcosms (HCS, Compost, Plant, Compost + Plant) and at the different sampling times (1, 133, and 224 d) are shown in Figure 2. PCBs 138, 153, 180, and the dioxin-like PCB 118 were generally the most abundant congeners found in all conditions. The congeners 77, 81, 123, 114, 169, and 189 were not found in the soil samples, possibly as they were not present or under the detection limits.

At day 224 the lower-chlorinated PCBs diminished by over half of their starting concentration ( $p < 0.01$ ) in all microcosms, including in the control ones (HCS), (Figure 2, Table 3). A sharp reduction ( $p < 0.05$ ) in the higher-chlorinated PCBs (Cl number > 4) was observed in Compost, Plant and Compost + Plant (Table 3) if compared to HCS. In the Compost and Compost + Plant treatments, congeners 105 and 156 decreased significantly more than in the Plant condition ( $p < 0.01$ ), (Figure 2).

In order to calculate the bioaccumulation factor (BAF), for defining the ratio of PCB amount in plant tissue linked to PCB amount in the soil, the analysis in plant tissues (leaves, stems) and roots was performed at the end of the experiment in the case of the Compost + Plant microcosms. Unfortunately, in the Plant microcosms, the Alfalfa died up a few days before the last sampling. This was because the plant was at the end of its life cycle, which was presumably extended when the compost was added to the soil (Compost + Plant microcosms).

**Table 2.** Probe sequences, target sites for each probe, and % formamide used (stringency).

Probe Name	Short Name	Specificity *	Sequence from 5' to 3'	Target Molecule and rRNA Position	Stringency (%)
ARCH915	ARCH	<i>Archaea</i>	GTGCTCCCCCGCCAATTCCT	16S rRNA, 915-934	20
EUB338 ** (EUB)	EUB	Most <i>Bacteria</i>	GCTGCCTCCCGTAGGAGT	16S rRNA, 338-355	20
EUB338 II **	EUB	<i>Planctomycetales</i>	GCAGCCACCCGTAGGTGT	16S rRNA, 338-355	20
EUB338 III **	EUB	<i>Verrucomicrobiales</i>	GCT GCC ACC CGT AGG TGT	16S rRNA, 338-355	20
ALF1b	A	$\alpha$ - <i>Proteobacteria</i> , some <i>Deltaproteobacteria</i> , <i>Spirochaetes</i>	CGT TCG (CT) TC TGA GCC AG	16S rRNA, 19-35	20
BET42a §	B	$\beta$ - <i>Proteobacteria</i>	GCC TTC CCA CTT CGT TT	23S rRNA, 1027-1043	35
GAM42a °	Γ	$\gamma$ - <i>Proteobacteria</i>	GCC TTC CCA CAT CGT TT	23S rRNA, 1027-1043	35
DELTA495a ^		Most $\delta$ - <i>Proteobacteria</i> , most <i>Gemmatimonadetes</i>	AGT TAG CCG GTG CTT CCT	16S rRNA, 495-512	35
DELTA495b ^	Δ	Some $\delta$ - <i>Proteobacteria</i>	AGT TAG CCG GCG CTT CCT	16S rRNA, 495-512	35
DELTA495c ^		Some $\delta$ - <i>Proteobacteria</i>	AAT TAG CCG GTG CTT CCT	16S rRNA, 495-512	35
CF319a	CF	Most <i>Flavobacteria</i> , some <i>Bacteroidetes</i> , some <i>Sphingobacteria</i>	TGG TCC GTG TCT CAG TAC	16S rRNA, 319-336	35
HGC69A	HGC	<i>Actinobacteria</i> (Gram-positive bacteria with high DNA G+C content)	TAT AGT TAC CAC CGC CGT	23S rRNA, 1901-1918	35
LGC354A **		<i>Firmicutes</i> (Gram-positive bacteria with low G+C content)	TGG AAG ATT CCC TAC TGC	16S rRNA, 354-371	35
LGC354B **	LGC		CGG AAG ATT CCC TAC TGC	16S rRNA, 354-371	35
LGC354C **			CCG AAG ATT CCC TAC TGC	16S rRNA, 354-371	35

\* Information from ProbeBase (<http://probase.csb.univie.ac.at>). \*\* The three EUB probes were mixed and used together; the three probes mix matched with 94% of the bacterial sequences available [43]. § The probe was used in combination with the unlabelled GAM42a competitor. ° The probe was used in combination with the unlabelled BET42a competitor. ^ The three DELTA probes were mixed and used together. \*\* The three LGC probes were mixed and used together to detect most *Firmicutes*.

**Table 3.** Removal percentages of the sum of PCB congeners grouped by number of Cl atoms at day 224 in the various treatments. HCS = historically contaminated soil; Compost = historically contaminated soil treated with compost; Plant = historically contaminated soil with Alfalfa plant; Compost + Plant = historically contaminated soil treated with the compost, in the presence of Alfalfa plant.

N. Chlorine	tri-Cl	tetra-Cl	penta-Cl	hexa-Cl	hepta-Cl
HCS	66% ± 0.66	66% ± 0.74	36% ± 0.00	2% ± 0.12	29% ± 0.01
Compost	60% ± 0.39	69% ± 0.24	52% ± 2.54	25% ± 0.69	46% ± 0.48
Plant	60% ± 0.73	70% ± 0.15	37% ± 5.98	20% ± 0.93	52% ± 1.16
Compost + Plant	54% ± 0.91	71% ± 0.23	53% ± 2.20	16% ± 1.32	44% ± 1.44

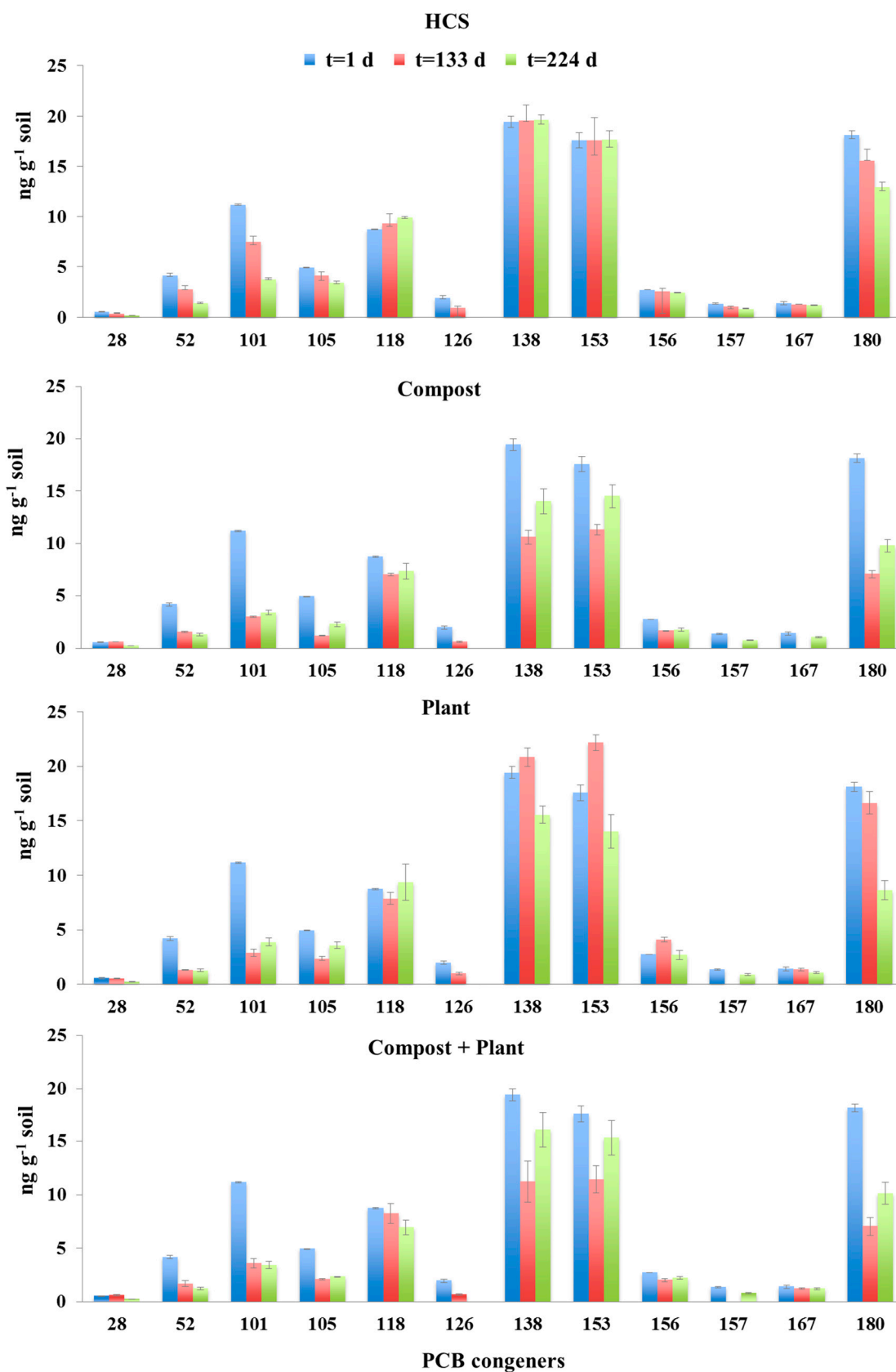
Various residual concentrations of some congeners (i.e., PCBs 28, 52, 118, 138, 153, and 180), were found in roots and shoots. The BAF values in roots were >1 in the case of PCB 28 (9.40), PCB 52 (2.28), and PCB 118 (1.29), while in the shoots only one congener (PCB 101) was found (BAF =1.33).

### 3.2. Microbial Abundance, Cell Viability and Dehydrogenase Activity

The compost effect on microbial abundance was immediate. Just one day after the compost addition, the microbial abundance was higher than in the conditions without compost (HCS and Plant). In HCS and Plant conditions the values were comparable to those found in the soil before the experiment set up. In fact, the cell number (No. cells g<sup>-1</sup> dry soil) increased significantly ( $p < 0.01$ ) in the microcosms where the compost was added (day 1) compared to the other conditions (Figure 3).

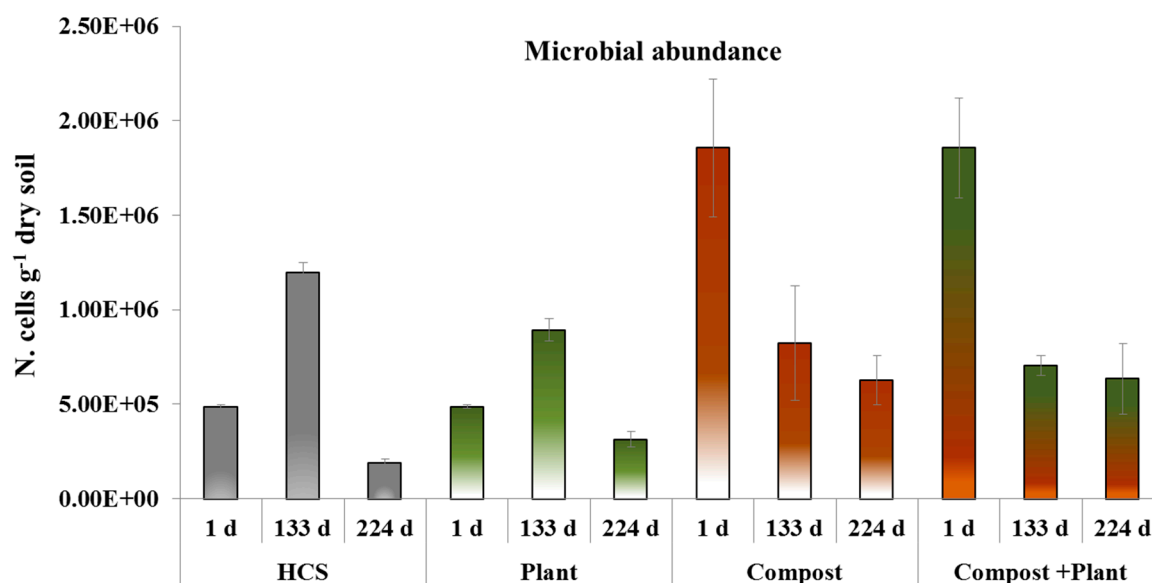
The values of cell viability at day 1 were lower in the compost microcosms (Compost and Compost + Plant) than those in the HCS and Plant microcosms, in line with the fact that dead bacteria were added with the compost (see the low cell viability value for compost in Table 1). A positive effect from the compost and plant presence was observed at day 224, with the highest cell viability value in this condition (Compost + Plant: 60% > Plant: 54% > Compost: 51% > HCS: 49%), as shown in Figure 4.

Adding compost immediately affected microbial activity (Figure 5). At day 1, the dehydrogenase values were in fact significantly higher ( $p < 0.01$ ) in the compost treated microcosms if compared with the HCS and Plant microcosms (91 µg TPF g<sup>-1</sup> for HCS and Plant vs 218 µg TPF g<sup>-1</sup> dry soil for Compost and Compost + Plant), while the plant enhanced the activity over time (Figure 5).

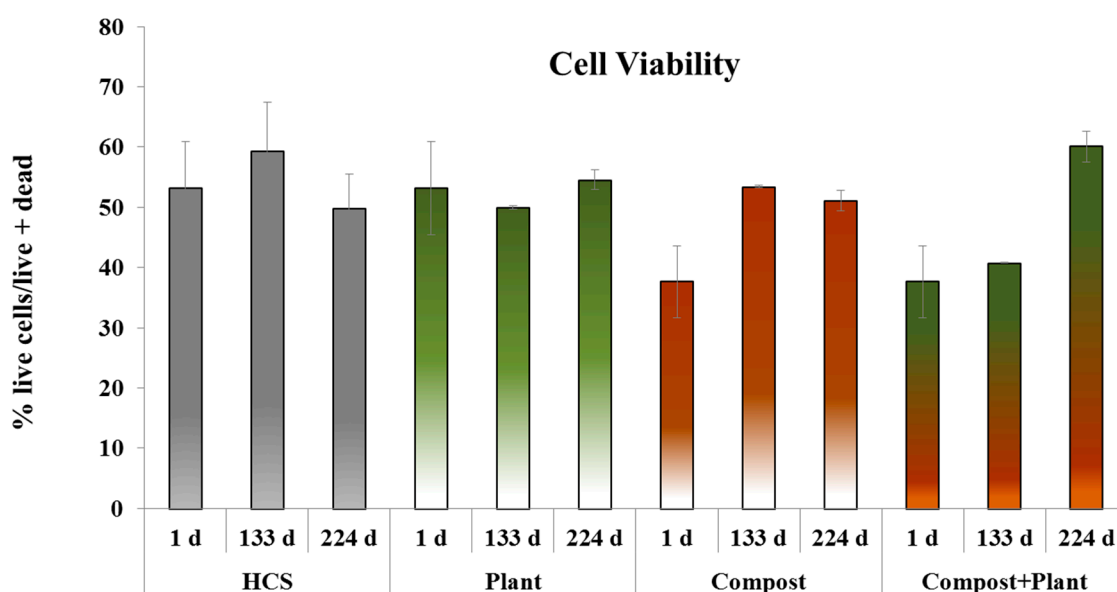


**Figure 2.** PCB congeners (ng g<sup>-1</sup> soil) analysed at different times (1, 133 and 224 d) in the soil microcosms. HCS: historically contaminated soil; Compost: historically contaminated soil amended with municipal solid waste compost; Plant: historically contaminated soil with *Medicago sativa* plant; Compost + Plant: historically contaminated soil amended with the compost, in the presence of *Medicago sativa* plant. The vertical bars represent the standard errors.

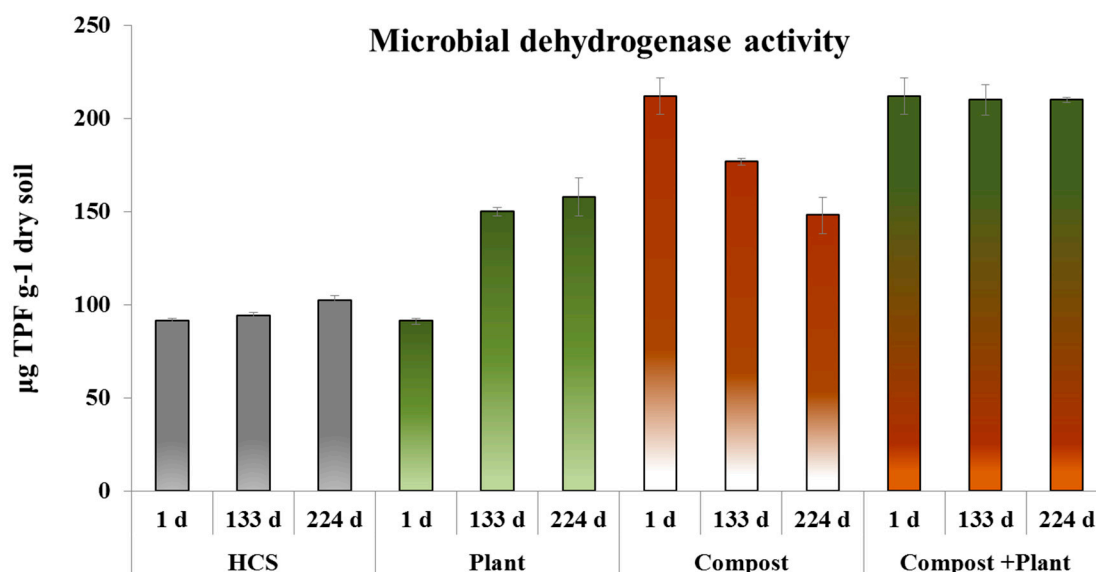




**Figure 3.** Microbial abundance (No. cells  $\text{g}^{-1}$  dry soil) in the three sampling times (days 1, 133 and 224). HCS: historically contaminated soil; Compost: historically contaminated soil amended with municipal solid waste compost; Plant: historically contaminated soil with *Medicago sativa* plant; Compost + Plant: historically contaminated soil amended with the compost, in the presence of *Medicago sativa* plant. The vertical bars represent the standard errors.



**Figure 4.** Cell viability (% live cells/live + dead) in the three sampling times (days 1, 133 and 224). HCS: historically contaminated soil; Compost: historically contaminated soil amended with municipal solid waste compost; Plant: historically contaminated soil with *Medicago sativa* plant; Compost + Plant: historically contaminated soil amended with the compost, in the presence of *Medicago sativa* plant. The vertical bars represent the standard errors.



**Figure 5.** Microbial dehydrogenase activity ( $\mu\text{g TPF g}^{-1}$  dry soil) over time (days 1, 133 and 224). HCS: historically contaminated soil; Compost: historically contaminated soil amended with municipal solid waste compost; Plant: historically contaminated soil with *Medicago sativa* plant; Compost + Plant: historically contaminated soil amended with the compost, in the presence of *Medicago sativa* plant. The vertical bars represent the standard errors.

Finally, the highest dehydrogenase values remained the same throughout the entire experimental period in only the Compost + Plant microcosms ( $p < 0.01$ ), showing the synergic and long-term positive effect adding compost and *M. sativa* on the microbial community of the rhizosphere.

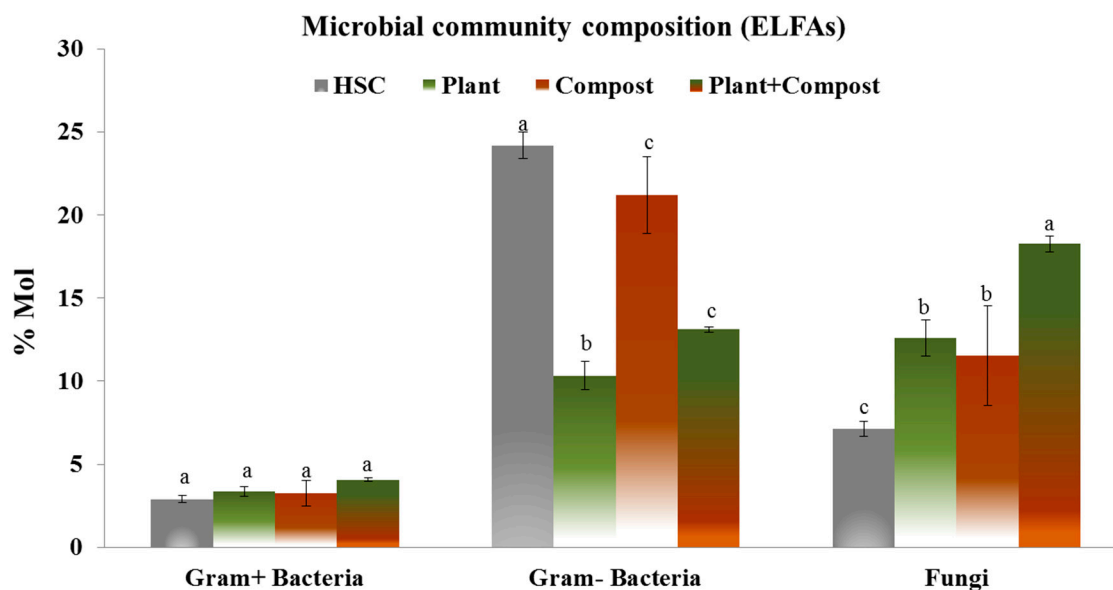
### 3.3. Microbial Community Composition Evaluated Using ELFAs

Eighteen individual ELFAs were identified and used to assess microbial biomass. Among them, the sums of the specific ELFAs detected were used as biomarkers: i15:0, a15:0, i16:0 and i17:0 were used for Gram-positive Bacteria, 16:1 $\omega$ 7; 18:1 $\omega$ 7c and 19:0cy for Gram-negative Bacteria and the sum of 18:1 $\omega$ 9c and 18:2 $\omega$ 6,9c for Fungi. The Fungi/Gram-negative ratio was significantly higher (at least two-fold) at day 224 than at day 1 in all microcosms, while Gram-positive Bacteria and the microbial biomass were not significantly influenced by the treatments during the experimental time (data not shown). At 224 days, similar percentages of Gram-positive bacteria were found in all treatments, while Gram-negative ones were significantly lower in the plant presence. Fungi were significantly higher in the condition where both the plant and compost were present than in the other conditions (Figure 6).

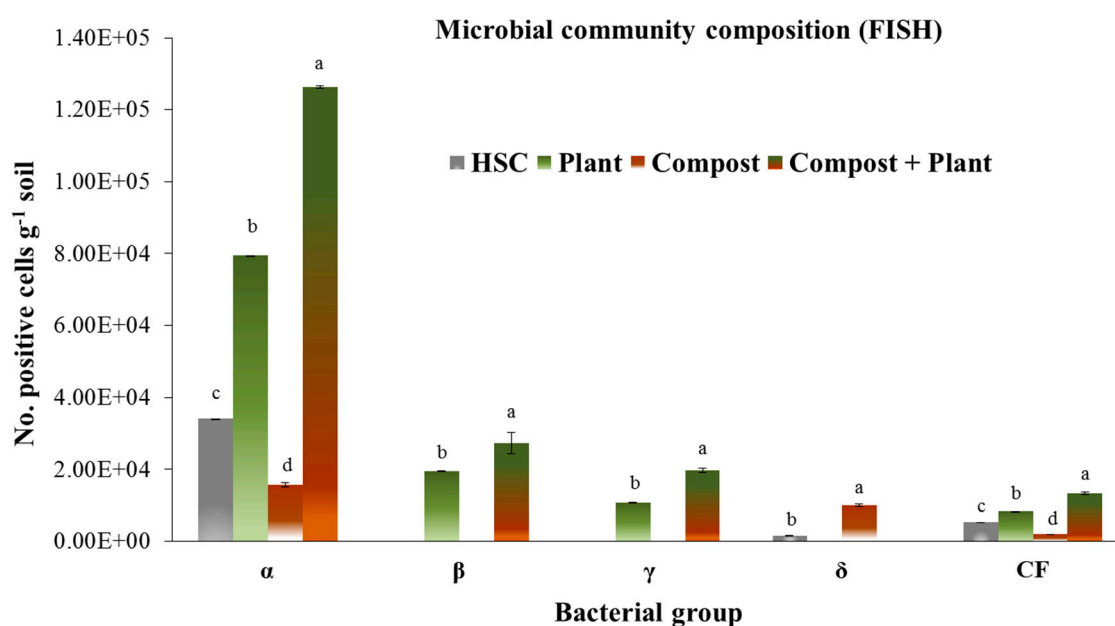
The fungal polyunsaturated fatty acids 18:1 $\omega$ 9c and 18:2 $\omega$ 6,9c to Bacteria (F/B) ratio and the iso to anteiso C15:0 biomarker (i/a) ratio showed the highest values of F/B together with the lowest values of i/a in the Plant and Compost + Plant conditions.

### 3.4. Microbial Community Structure Analysed Using FISH

At day 1, the Bacteria domain had a percentage of  $65.6 \pm 3.9\%$  ( $6.4 \times 10^5$  cells  $\text{g}^{-1}$  soil). Inside this domain, the most abundant group was  $\beta$ -Proteobacteria ( $1.6 \times 10^5$  cells  $\text{g}^{-1}$  soil) then  $\alpha$ -Proteobacteria ( $5.1 \times 10^4$  cells  $\text{g}^{-1}$  soil), *Bacteroidetes* ( $3.8 \times 10^4$  cells  $\text{g}^{-1}$  soil),  $\delta$ -Proteobacteria ( $2.5 \times 10^4$  cells  $\text{g}^{-1}$  soil) and  $\gamma$ -Proteobacteria ( $1.3 \times 10^4$  cells  $\text{g}^{-1}$  soil). Neither Archaea, nor Gram-positive bacteria (both HGC and LGC) were detected. At the end of the experiment a shift ( $p < 0.01$ ) in the dominance of some bacterial groups was observed in the presence of the plant. In particular,  $\alpha$ -Proteobacteria significantly increased in the Plant and Compost + Plant microcosms, while  $\beta$ -Proteobacteria reduced drastically and  $\delta$ -Proteobacteria were never detected (Figure 7).



**Figure 6.** Microbial community structure assessed by ester-linked fatty acids (ELFA) expressed as % Mol at day 224 in the different treatments. HCS: historically contaminated soil; Compost: historically contaminated soil amended with municipal solid waste compost; Plant: historically contaminated soil with *Medicago sativa* plant; Compost + Plant: historically contaminated soil amended with the compost, in the presence of *Medicago sativa* plant. The vertical bars represent the standard errors. Lowercase letters indicate significant differences ( $p < 0.05$ ) among treatments within the same microbial group.



**Figure 7.** Microbial community structure assessed at day 224 in the various treatments by Fluorescence In Situ Hybridization (FISH), expressed as number of positive cells (No. positive cells  $g^{-1}$  soil). HCS: historically contaminated soil; Compost: historically contaminated soil amended with municipal solid waste compost; Plant: historically contaminated soil with *Medicago sativa* plant; Compost + Plant: historically contaminated soil amended with the compost, in the presence of *Medicago sativa* plant.  $\alpha$ :  $\alpha$ -Proteobacteria;  $\beta$ :  $\beta$ -Proteobacteria;  $\gamma$ :  $\gamma$ -Proteobacteria;  $\delta$ :  $\delta$ -Proteobacteria; CF: *Cytophaga-Flavobacterium-Bacteroidetes* (CFB group). Lowercase letters indicate significant differences ( $p < 0.05$ ) among treatments within the same microbial group.

#### 4. Discussion

PCB environmental contamination is a very complex issue, mainly because many different congeners are simultaneously present, and each congener can be transformed into another one by the addition/removal of one or more chlorine atoms. The resistance of PCBs to biodegradation varies in line with the molecular structure complexity, soil aging, texture, biological diversity and abundance, and abiotic factors affecting microbial activity (e.g., temperature, oxygen, and electron donors), [10,22,44].

The decrease in PCBs observed in both treated and untreated microcosms (although significantly lower in the latter ones) demonstrated the natural capability of the microbial autochthonous community in the soil studied to transform these molecules. At the end of the experiment, in fact, PCB28 and PCB52 decreased by more than 50% of their initial concentrations in all soils, including the untreated HCS. The decrease in lower halogenated PCBs, and in particular, of the 28 and 52 congeners, was probably due to the soil handling (e.g., sieving, watering) before the experiment set up, that stimulated aerobic microbial degradation. On the contrary, the degradation of higher-chlorinated PCBs (in particular 105, 138, 156, and 180) was positively influenced by the plant and/or compost presence. The highest removal values for the penta, hexa, and hepta-Cl congeners, observed in soil treated with compost, plant, and compost plus plant, showed that these treatments were able to promote, although at different times and presumably in different ways, the bio-transformation of the higher-chlorinated PCBs, by activating both aerobic and anaerobic degradation pathways. Compost can be a carbon source for anaerobic dechlorination and, where co-present, can stimulate plant growth; the rhizosphere can directly stimulate PCB degradation through its exudates and indirectly provide a carbon source and/or microhabitat suitable for increasing microbial activity [45]. The increase (day 133) and subsequent decrease (day 224) in PCBs 138, 153, and 180 with a plant presence could be due to the decrease in the octo-chlorinated PCB194, which was indeed found in a high concentration ( $23 \mu\text{g kg}^{-1}$  soil) in the initial field site characterization and was later not found. PCB194 is in fact reported to be transformed into PCB180, and the latter in turn into PCB138 or 153 (hexa-Cl). PCB153 can be transformed into PCB101 (penta-Cl) and the latter into PCB52 (tetra-Cl) [46,47]. In similar experiments, the relative increase in some PCBs in aged soil with a plant presence was explained by the fact that root exudates can act as surfactants, binding PCBs and increasing their bioavailability in soil [27,48–50]. In any case, whatever the process involved, at day 224 the final PCB concentrations were lower than the initial ones, showing not only the effectiveness of *Medicago sativa* in stimulating their transformation, but also its positive effects on the structure and functioning of the soil natural microbial community. Compost and plants can directly and indirectly increase microbial diversity and stimulate microbial activity and contaminant degradation. The positive effect of applying compost on soil is ascribable to the presence of mineral and humidified organic matter, which can improve the soil aggregation and porosity and increase microbial activity and biomass [51–53]. The addition of organic matter through composted biosolid waste and planting of vegetation are strategies suited to the recovery and improvement of soil affected by erosion and/or contamination. In line with our results, other works report that Alfalfa plays a key role in promoting PCB dechlorination [29,54]. Furthermore, autochthonous microbial communities in PCB contaminated and aged soil can enhance the breakdown of PCBs in the rhizosphere [10,45,55–58]. Additionally, the rhizosphere and compost can serve as nutrient hotspots with a high microbial abundance, which promotes horizontal gene transfer [59].

The BAF values  $> 1$  found in roots in the case of the lower-chlorinated congeners (PCB 28 and 52) are in line with the results found with other plant species [60,61]. Owing to high hydrophobicity, PCB translocation through the transpiration stream of the plant is improbable. Moreover, the process of translocation relies on the ability of plants to take contaminants from the soil together with water and nutrients. This depends on the intrinsic chemical characteristics, such as solubility,  $K_{ow}$  value etc. However, in some cases PCBs transportation in plants may occur if root exudates act as surfactants forming more hydrophilic complexes, as in the case of PCB 101, found in shoots in Compost + Plant microcosms. In any case, these complex interactions still have to be better investigated [22,60,61].

In this study, dehydrogenase activity was positively influenced by the treatments and particularly by the co-presence of compost and the plant, with a significant increase that was stable over a long period, in line with previous studies [33,34,62].

Similarly, the composition of the microbial community was positively influenced by the plant. In fact, an increase in the Fungi to Gram-negative Bacteria ratio was observed, in accordance with other studies. In particular Fungi are reported to improve soil quality even through positive modifications of the bacterial community [63,64].

The highest increase in Fungi observed in the Compost+Plant microcosms was in line with the reported *Medicago sativa* symbiotic interactions with these microorganisms [65].

The iso to anteiso C15:0 biomarker (i/a) ratio is considered a soil quality and chemical stress indicator [63,66,67]. Therefore, the highest value of i/a ratio found in HCS suggested a low soil quality ascribable to PCB presence and rhizosphere absence. ELFA analysis is a valuable culture-independent method for an overall description of community structure [68]. However, it produces a low taxonomic resolution [69]. The FISH analysis was therefore performed to investigate a little bit more in detail, the changes in microbial structure. The FISH results made it possible to identify which bacterial groups were positively affected by the Plant or compost or simultaneously by Plant and compost occurrence, i.e.,  $\alpha$ -,  $\beta$ -,  $\gamma$ -*Proteobacteria*. *Proteobacteria* include most species engaged in the main biogeochemical cycles and are the most abundant phylum present in a good quality state soil [39,70,71] and/or a soil with rhizosphere formation [39,72,73]. Lastly, the dominance and increase in  $\alpha$ -*Proteobacteria*, which includes many genera of nitrogen-fixing bacteria, can be ascribed to the Alfalfa capacity to support nitrogen fixing bacteria growth.

Finally, as regards the species that can be involved in PCB transformation, among  $\alpha$ - (e.g., *Spingomonas*),  $\beta$ - (e.g., *Burkholderia*, *Alcaligenes*, *Achromobacter*, *Comamonas*, *Ralstonia*), and  $\gamma$ -*Proteobacteria* (e.g., *Pseudomonas*, *Acinetobacter*), there are species reported to be able to degrade PCBs [74,75]. In some cases, the apparent discrepancy between the ELFA and FISH data for Gram-positive Bacteria was presumably because the FISH technique detects active bacteria with an adequate number of ribosomes [39,40], while the ELFA one produces a phenotypic fingerprinting of microbial communities, whatever their activity.

## 5. Conclusions

The synergic effects of adding compost and plants in increasing soil quality in terms of microbial activity and structure, together with the degradation of the higher-chlorinated congeners of PCBs, were demonstrated. These results encourage the use of plant-assisted bioremediation in promoting PCB transformation. Further studies are needed to investigate the detailed pathways of microbial degradation of PCBs in the rhizosphere and for isolating bacterial strains that can be applied for bioaugmentation purposes.

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